# Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions

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## Summary

• Arbuscular mycorrhizal (AM) fungi are important below-ground carbon (C) sinks that can be sensitive to increased nitrogen (N) availability. The abundance of AM fungi (AMF) was estimated in maple (*Acer* spp.) fine roots following more than a decade of experimental additions of N designed to simulate chronic atmospheric N deposition.

• Abundance of AMF was measured by staining and ocular estimation, as well as by analyzing for the AMF indicator fatty acid 16:1ω5c in phospholipid (biomass indicator) and neutral lipid (lipid storage indicator) fractions.

• Arbuscular mycorrhizal fungal biomass, storage structures and lipid storage declined in response to N addition measured by both methods. This pattern was found when AM response was characterized as colonization intensity, on an areal basis and in proportion to maple above-ground biomass. The phospholipid fraction of the fatty acid 16:1ω5c was positively correlated with total AMF colonization and the neutral lipid fraction with vesicle colonization.

• Decreased AMF abundance with simulated N deposition suggests reduced C allocation to these fungi or a direct soil N-mediated decline. The fatty acid (phospholipid and neutral lipid fractions) 16:1ω5c was found to be a good indicator for AMF active biomass and stored energy, respectively.

**Key words:** *Acer saccharum* (sugar maple), arbuscular mycorrhizal fungi (AMF), neutral lipid fatty acid (NLFA), nitrogen deposition, phospholipid fatty acid (PLFA), staining.

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## Introduction

Human activities have dramatically increased atmospheric nitrogen (N) deposition across large regions of the world, and rates of atmospheric N deposition are still increasing (Galloway *et al.*, 1994; Vitousek *et al.*, 1997). Deposition rates can exceed 75 kg N $ha^{-1}$ $yr^{-1}$ in regions downwind of large industrial complexes or intensive agriculture in the US and Europe (Dise & Wright, 1995; Cunha *et al.*, 2002). Within the US, the northeastern region is the area with the highest rates of N deposition, with values routinely exceeding 10 kg N $ha^{-1}$ $yr^{-1}$ (NADP, 2006). Atmospheric N deposition can have a significant impact on natural ecosystems by increasing acidification and eutrophication, leading to a decrease in biodiversity and altered ecosystem function (Vitousek *et al.*, 1997).

Mycorrhizal fungi make up a large part of the microbial biomass in terrestrial ecosystems and are important suppliers of nutrients to plants, especially in nutrient-poor environments such as boreal and temperate forests (Smith & Read, 1997). Increasing amounts of N deposition could therefore have a potential effect on the presence and functioning of

mycorrhizal fungi. Increased atmospheric N deposition generally has a negative effect on the abundance of mycorrhizal fungi (Wallenda & Kottke, 1998; Treseder, 2004). However, most studies in temperate forests have involved ectomycorrhizal fungi. Arbuscular mycorrhizal (AM) fungal abundance in temperate deciduous forests has been examined in only two multiyear N-fertilization studies (Hutchinson *et al.*, 1998; Lansing, 2003), with a range of results reported for different tree species. Both of these studies used N addition amounts much higher than anthropogenic atmospheric N deposition and were carried out for a maximum of 4 yr, so there is a need for investigations covering longer periods at realistic deposition rates.

The Michigan Gradient Study offers a rare opportunity to study the long-term effects of altered N-availability on AM fungi (AMF) in the roots of maple trees (*Acer* spp.). This study has applied experimentally simulated N deposition since 1994 to *Acer saccharum* (sugar maple)-dominated hardwood forests. Sugar maple belongs to a dominant temperate tree genus forming a symbiotic relationship solely with AMF. Previous results in this study have shown a change in stand-level C allocation across sites. Of particular interest in the present context, N-amended plots showed decreased soil respiration (Burton *et al.*, 2004) not explained by changes in root biomass or root respiration (Burton *et al.*, 2004), or in microbial biomass or microbial respiration in the mineral soil (Zak *et al.*, 2006). Therefore the present study focuses on the intraradical response of AMF, which can be direct recipients of a significant fraction of net primary production and are known to be responsive to N inputs. The abundance of extraradical hyphae, the other active pool, will be addressed in a subsequent study.

The abundance of AMF has traditionally been quantified microscopically using stained roots (% root colonization), estimation of extraradical hyphal length and/or spore counts. These techniques are time-consuming and can be subjective; they also do not provide reliable estimates of living biomass (Allison & Miller, 2004). Fatty acid (phospholipid and neutral lipids) analysis has recently been used to quantify AMF. These have a much higher concentration of the fatty acid 16:1ω5c than other fungi and this fatty acid is not present in plant cells. It has been successfully used as an indicator of AMF abundance within both soil and plant roots (Olsson, 1999; Olsson & Johansen, 2000). The phospholipid fatty acid (PLFA) 16:1ω5c has been found to be a good measure of live AMF biomass (hyphae, arbuscules, coils and vesicles) and the neutral lipid fatty acid (NLFA) 16:1ω5c measures stored lipids within AMF storage structures such as vesicles and spores (Olsson, 1999).

The first objective of the present study was to examine the potential effects of realistic amounts of simulated N deposition over 12 yr on the abundance of AMF in the roots of maples. We hypothesized that the abundance and energy storage of AMF and host proportional allocation to intraradical AMF would decline in response to N addition, which could explain the observed decline in soil respiration across the sites. The second objective was to perform a comparison between traditional staining and lipid analysis techniques. Specifically, we compared the percentage of roots colonized by stained AMF and the concentration of PLFA 16:1ω5c as estimators of total AM fungal root colonization; and number of vesicles vs NLFA 16:1ω5c as estimators of AMF stored energy.

## Materials and Methods

### Site description and sampling

The Michigan Gradient Study consists of four study sites located in sugar maple-dominated forests throughout Michigan, USA (Fig. 1). Sugar maple (*Acer saccharum* Marshall) accounts for > 80% of the total stand basal area at these sites (Pregitzer *et al.*, 2004). Each site consists of six 30 × 30 m plots, with three plots at each study site receiving N amendment and the other three untreated. N additions have been applied annually as six 5 kg N increments of $NaNO_3$ during the growing season since 1994, for a total of 30 kg N $ha^{-1}$ $yr^{-1}$. Ambient N deposition at these sites in 2004 ranged from 4.8 kg N $ha^{-1}$ $yr^{-1}$ at the northernmost site to 8.3 kg N $ha^{-1}$ $yr^{-1}$ at the southernmost site (NADP, 2006). From north to south along the gradient, the mean annual temperature increases from 4.8 to 7.6°C, with a concurrent increase in the length of the growing season. Soil development, stand age and plant composition are similar among the sites. More detailed information on the sites can be found in Burton *et al.* (1991).

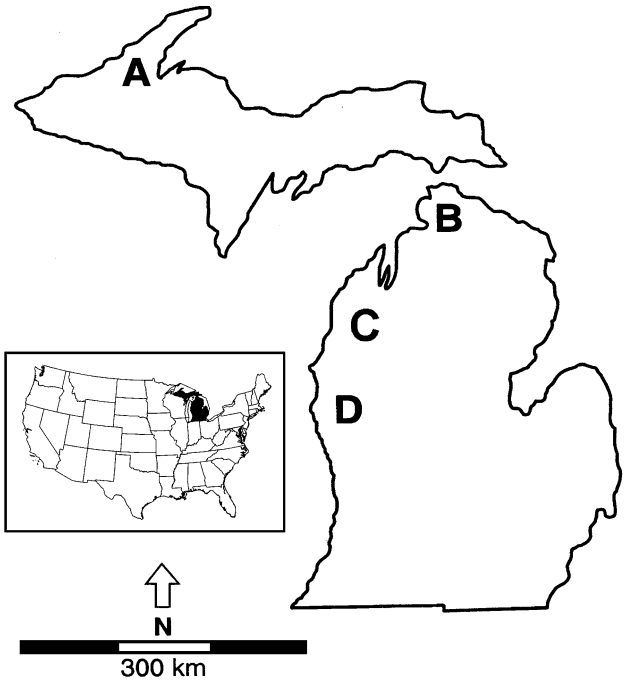


**Fig. 1** Locations of the study sites (A–D) in Michigan. The inset shows the location of Michigan (filled area) within the USA.

All the plots were divided into six equally sized subplots (10 × 15 m) for soil sampling. One soil core, 2 cm diameter and 10 cm deep, was taken randomly in each subplot in July and October 2005. Each soil core was washed through a 2 mm and 1 mm screen until most soil particles were washed out. The remaining roots in both screens were handpicked for maple roots, easily recognized by their unique beaded structure (Pregitzer *et al.*, 2002). Because the distinction between red maple (*Acer rubrum*) and sugar maple roots cannot be made easily, some samples might contain minor quantities of red maple roots. However, the percentage of red maple in the plots averaged only 7% of the total basal area and there were no differences between treatments in the percentage of red maple.

After sorting, the roots were cleaned from residual soil particles by sonication for 5 min. After cleaning, all maple roots were scanned (HP Scanjet ADT 6300C series) for further specific root length analysis (cm $g^{-1}$ root; WinRhizo Pro, 2005b, Regent instruments Inc., Quebec, Canada). The maple roots were divided into two diameter classes, fine roots (≤ 0.5 mm) and larger roots (> 0.5 mm), and root weights were recorded for both diameter classes. In each core, the maple fine roots were divided into three subsamples. One subsample was frozen and freeze-dried (Labconco, Freezone 4.5) for molecular community analysis (not discussed in this paper), and the other two subsamples were each composited at the plot level to be used in AM fungal colonization and lipid analyses.

## AM fungal colonization

The first composite sample was used to measure the AMF colonization of maple roots using a trypan blue (TB) staining technique after Koske & Gemma (1989). Approximately 0.25 g of fresh roots were placed in a 10% KOH solution and heated to 90°C using a water bath until sufficiently cleared. To increase uptake of the staining solution, the cleared roots were slightly acidified by submerging them in an HCl (0.5 N) solution for 5 min. The roots were then stained overnight in a TB solution (0.6 g TB in 1 l of 1 : 2 : 2 (v : v : v) lactic acid : glycerol : deionized water). To remove excess stain, the roots were first rinsed in deionized water and destained in a 1 : 2 : 2 (v : v : v) lactic acid : glycerol : deionized water solution.

Stained roots were permanently mounted on microscope slides in three parallel rows with polyvinyl alcohol-lactoglycerol (PVLG) and colonization was measured at 200× magnification using the line intersect method of McGonigle *et al.* (1990). Three slides were analyzed per sample, measuring AM fungal colonization at 50 intersects per slide by the presence of hyphae, vesicles, spores, arbuscules and/or coils. Total AMF colonization is defined as occurrence of AM intraradical hyphae, arbuscules, coils, vesicles and intraradical spores. Spores and vesicles had similar morphology, so their occurrences were summed and will be referred to as vesicles.

## Lipid extraction and analysis

The second composite fine root sample was frozen, freeze-dried (Labconco, Freezone 4.5), weighed and ground (SPEX Certiprep Mill, 8000 M). Lipids were extracted from approx. 15 mg of ground roots vortexed in a one-phase mixture of methanol, chloroform and phosphate buffer (pH 7.4) with a volume ratio of 2 : 1 : 0.8, using the Frostegård *et al.* (1991) modification of the Bligh & Dyer (1959) method. The extracted lipids were separated into neutral-, glyco-, and phospholipid fractions by silicic acid column chromatography, by eluting with chloroform, acetone and methanol, respectively. The neutral lipids and phospholipids were methylated to free fatty acid methyl esters (FAMEs) using a mild alkaline solution. The FAMEs were then dissolved in hexane and analyzed by gas chromatography using a 6890 N GC (gas chromatograph; Agilent Technologies, Palo Alto, CA, USA) with a Ultra 2 column (30 m, 0.2 m ID, 0.33 μm film), a FID detector and hydrogen as carrier gas. Conditions (temperature and time) for the GC analyses are set by the Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE, USA). The FAME 19 : 0 (Matreya Inc., State College, PA, USA) was used as internal quantitative standard and the FAMEs were identified by a Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE, USA) using a eukaryotic mix (MIDI Inc.) as qualitative standards. To confirm MIDI software identification, several samples were also analyzed using a GC-MS (gas chromatograph-mass spectrometer) at Argonne National laboratory (R. M. Miller laboratory) in parallel with the analysis using the GC with MIDI software. The fatty acid 16:1ω5c was used as an indicator for AMF (Olsson, 1999; Olsson & Johansen, 2000).

## Calculations of AMF abundance

Arbuscular mycorrhizal fungi root colonization can be calculated on different bases, each of which sheds light on different aspects of response to treatments. The most commonly used metric is colonization as a percentage of root length (staining method) or fatty acid biomass as a proportion of root biomass (fatty acid method), which sheds light on treatment effects on the colonization intensity of AMF in roots, but not on the net changes in fungal abundance at the stand level, or proportional allocation to mycorrhizas. Fungal colonization on an area or soil volume basis sheds light on cumulative treatment effects on fungal abundance mediated by both colonization intensity and variations in length and biomass of host roots in response to treatments; these methods are useful for defining treatment response of fungal biomass at the stand level. Expression of fungal colonization as a proportion of host above-ground (woody and litter) biomass sheds light on the proportional biomass allocation of maples to AMF, which is of interest to ecosystem modelers.

For calculation of maple AMF abundance per $cm^3$ of soil, referred to as stand-level maple AMF colonization, the following equations were used:

for ocular (root staining) data:
(cm AMF root length $cm^{-3}$ soil) = (% AMF colonization) × (cm root length $g^{-1}$ root) × (g root $cm^{-3}$ soil)

for fatty acid data: (nmol 16:1ω5c $cm^{-3}$) =
(nmol 16:1ω5c $g^{-1}$ root) × (g root $cm^{-3}$ soil)

To determine the effect of treatments on maple proportional allocation to AMF biomass, the AMF abundance was expressed in relation to maple above-ground biomass:

for ocular data:
(cm AMF root length $g^{-1}$ maple biomass) = (cm AMF root length $m^{-2}$ soil)/(g maple biomass $m^{-2}$ soil)

for fatty acid data: (nmol 16:1ω5c $g^{-1}$ maple biomass) =
(nmol 16:1ω5c $m^{-2}$ soil)/(g maple biomass $m^{-2}$ soil)

The (cm AMF root length $m^{-2}$ soil) and (nmol 16:1ω5c $m^{-2}$ soil) parameters represent the values per $m^2$ obtained from the top 10 cm of soil. Maple above-ground biomass (woody plus litter) has been measured annually since 1988 at all sites using the methods described in Reed *et al.* (1994) and Burton *et al.* (1991).

### Statistical analysis

Differences in dependent variables (root colonization, vesicle colonization, PLFA 16:1ω5c and NLFA 16:1ω5c) between treatments were determined using a two-way repeated-measures ANOVA with N treatment ($n = 2$) and site ($n = 4$) as factors and the sample date ($n = 2$) as repeated measures. Transformations (square root, natural logarithm and arcsine) were applied as appropriate to ensure a normal distribution and equal variances. Given significant time effects, site effects and (in some cases) treatment–site interaction terms, we examined site-level effects using univariate ANOVA with N-treatment as a fixed factor. For each date, the relationships between total AMF colonization and PLFA 16:1ω5c, and between vesicle colonization and NLFA 16:1ω5c, were analyzed using linear regression analysis ($n = 24$ per sample date).

## Results

### Root staining

Nitrogen addition led to significantly reduced total AM fungal structures (Fig. 2a,b) and exchange structures (arbuscules + coils) (Fig. 2e,f) for all three metrics (colonization intensity, stand-level maple AMF abundance, and proportional allocation to AMF) (Table 1). Other individual structures (hyphae, vesicles) showed similar, but weaker, trends of decline in response to N addition that were significant for only a subset of metrics (Table 1, Fig. 2c,d,g,h).

Changes from July to October were common for multiple metrics and structures (Table 1). For total AMF structures, significant growing season increases occurred for colonization intensity and stand-level abundance (Table 1). For hyphae (Fig. 2c,d) and exchange structures (Fig. 2e,f) the seasonal effect was significant for stand-level abundance and proportional allocation with various directions of change. For vesicles a significant growing season increase was observed only using the colonization intensity metric (Table 1).

Significant site effects were seen for all AMF structures using all metrics, with the main trend showing a decrease in total AMF abundance and vesicles in both treatments from site A–C (most metrics) or site A–D (Fig. 2a,b,g,h) (Table 1). This decrease was most apparent for vesicles (Fig. 2g,h), and not observed with hyphae or exchange structures (Fig. 2c,d and 2e,f, respectively).

There were also some significant treatment–site and site–time interactions. Treatment–site interactions were strongest and most consistent across metrics for exchange structures and, to a lesser extent, for vesicles and total AMF structures (Table 1). Variation in the strength of the response to N addition was evident at the site level, with strongest effects at site C and weakest effects at site D (Fig. 2). Site–time interactions were seen in total AMF structures, exchange structures and hyphae for all three metrics, with site D again diverging from other sites for total AMF structures (Fig. 2a,b) and exchange structures (Fig. 2e,f) and site B diverging for hyphae (Fig. 2c,d) (Table 1). There were no significant treatment–time or treatment–site–time interactions for any variables (data not shown).

### Lipid analysis

Nitrogen addition led to a decrease in AMF indicator PLFAs and NLFAs for all metrics (Table 1, Fig. 3). A significant growing season increase was evident for PLFA 16:1ω5c only for the colonization intensity metric (Fig. 3a,b), but for NLFAs the increase was evident with all metrics (Table 1, Fig. 3e,f,g,h). Differences in PLFA 16:1ω5c among sites only became apparent when PLFA 16:1ω5c was expressed at the stand level or as proportional allocation (Table 1, Fig. 3c,d), whereas differences among sites in NLFA 16:1ω5c were evident for all metrics (Table 1, Fig. 3e,f,g,h). As for staining, there was some variation in the strength of the treatment effect among sites, although no significant interaction terms were found for PLFAs or NLFAs (Table 1, Fig. 3).

### Relationship of root staining and lipid analysis

Phospholipid fatty acid 16:1ω5c content had a significant positive linear relationship with total AMF colonization in



 

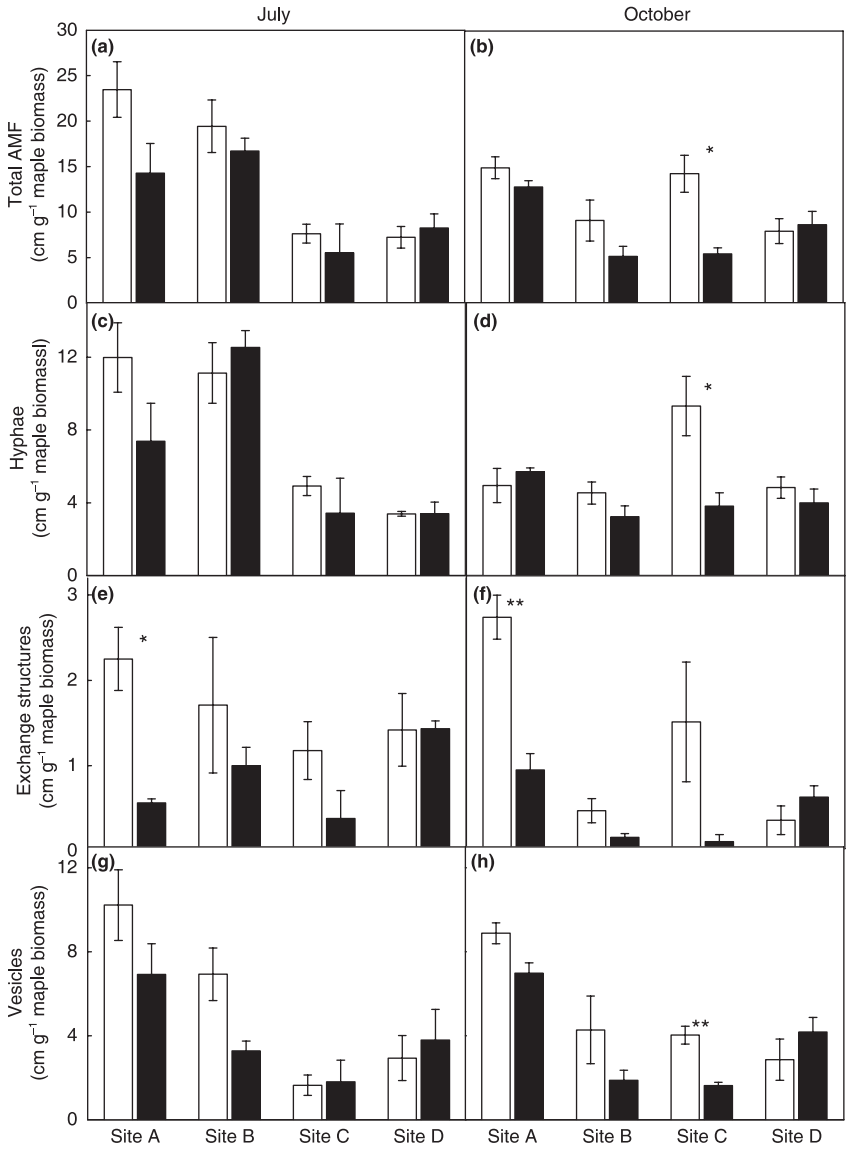


**Fig. 2** Mean proportional allocation to arbuscular mycorrhizal fungi (AMF) in maple (*Acer* spp.) fine roots for the four study sites by treatment (open bars, control; closed bars, N-amended) for July and October 2005: total (a, b), hyphal (c, d), exchange structures (e, f) and vesicles (g, h). Error bars indicate 1 SE of the mean. All AMF structures showed a significant overall N treatment and site effect (see Table 1 for details). Means of N treatments differ significantly at site: *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$.

October ($R^2 = 0.39$, $P = 0.001$) and a similar trend in July ($R^2 = 0.14$, $P = 0.07$) (Fig. 4a). NLFA 16:1ω5c showed a strong positive linear relationship with the percentage vesicle colonization ($P < 0.0001$) on both sampling dates ($R^2 = 0.59$ (July) and $R^2 = 0.67$ (October), Fig. 4c). Although the percentage vesicle colonization had an equal range on both sampling dates, the amount of NLFAs for a particular percentage of vesicle colonization was much higher in October ($y = 1.47 + 0.41x$) than in July ($y = 0.67 + 0.09x$) (Fig. 4c). This large increase in NLFA 16:1ω5c content with a similar percentage of vesicles is very apparent in the almost fivefold increase of the October regression line slope compared with the July slope (0.41 and 0.09, respectively). When expressed on a maple stand level, both the relationships between PLFA 16:1ω5c vs AMF root colonization and NLFA 16:1ω5c vs vesicle colonization became stronger (Fig. 4b,d).

## Discussion

### Nitrogen effects on temperate forest AMF

The decrease in AMF root colonization with N addition is very apparent from the results using both techniques. This is the first study to demonstrate such a response in temperate hardwood forests treated for so long with realistic amounts of simulated N deposition. Hutchinson *et al.* (1998) found a significant decrease in percentage AMF colonization of sugar maple roots at one site after 3 yr of 1000 kg $ha^{-1}$ $yr^{-1}$ N addition, while another site showed no difference after 2 yr of N addition. Lansing (2003) also found a reduction in AMF colonization rates for sugar maple after 4 yr of 100 kg $ha^{-1}$ $yr^{-1}$ N addition. Interestingly Lansing's reduction in AMF colonization for sugar maple in Michigan was similar to that found

 

New Phytologist

**Table 1** Comparison of *P*-values for all quantification methods and metrics of estimation of mycorrhizal fungal (AMF) colonization of maple (*Acer* spp.) roots across a nitrogen deposition gradient in Michigan, USA

| Metric | Method | Structures | Treatment | Site | Time | Treatment × site | Site × time |
|---|---|---|---|---|---|---|---|
| AMF root colonization intensity | Staining | Total AMF | 0.002 | 0.001 | 0.001 | 0.26 | 0.003 |
| | Staining | Hyphae | 0.05 | 0.03 | 0.08 | 0.18 | < 0.001 |
| | Staining | Exchange structures | < 0.001 | 0.008 | 0.08 | 0.01 | 0.004 |
| | Staining | Vesicles | 0.03 | < 0.001 | < 0.001 | 0.04 | 0.81 |
| | Fatty acid 16:1ω5c | Phospholipid | 0.005 | 0.55 | < 0.001 | 0.43 | 0.97 |
| | Fatty acid 16:1ω5c | Neutral lipid | 0.007 | 0.005 | < 0.001 | 0.27 | 0.85 |
| Stand-level maple AMF abundance | Staining | Total AMF | 0.02 | < 0.001 | 0.03 | 0.05 | 0.001 |
| | Staining | Hyphae | 0.06 | 0.01 | 0.03 | 0.10 | < 0.001 |
| | Staining | Exchange structures | 0.001 | 0.007 | 0.003 | 0.006 | 0.006 |
| | Staining | Vesicles | 0.06 | < 0.001 | 0.93 | 0.03 | 0.06 |
| | Fatty acid 16:1ω5c | Phospholipid | 0.01 | 0.001 | 0.24 | 0.14 | 0.07 |
| | Fatty acid 16:1ω5c | Neutral lipid | 0.02 | < 0.001 | < 0.001 | 0.07 | 0.22 |
| Proportional allocation to AMF | Staining | Total AMF | 0.007 | < 0.001 | 0.11 | 0.08 | 0.001 |
| | Staining | Hyphae | 0.02 | 0.007 | 0.03 | 0.19 | < 0.001 |
| | Staining | Exchange structures | < 0.001 | 0.009 | 0.003 | 0.01 | 0.006 |
| | Staining | Vesicles | 0.05 | < 0.001 | 0.93 | 0.07 | 0.06 |
| | Fatty acid 16:1ω5c | Phospholipid | 0.004 | 0.001 | 0.27 | 0.21 | 0.07 |
| | Fatty acid 16:1ω5c | Neutral lipid | 0.01 | < 0.001 | < 0.001 | 0.12 | 0.22 |

in our study (*R*-values of 0.88 and 0.80, respectively), where *R* is the response ratio (*R* = mean of treatment divided by mean of control) (Treseder, 2004). Our total N addition over 12 yr (360 kg $ha^{-1}$) was comparable to their total N addition over 4 yr (400 kg $ha^{-1}$).

Several factors could be causing the reduction of the AM symbiont in an N-amended environment. One hypothesis is that N addition reduces host C allocation to AMF. This is consistent with the significant results found in the analyses of proportional allocation to AMF by the maples (above-ground and litter biomass), which is lower in the N-amended plots. The high N deposition sites (sites C and D) also had a lower proportional allocation to AMF independent of treatment, which might be the result of long-term differences in ambient N deposition. If less C is being allocated to the fungal symbiont this could also explain some of the reduced soil respiration found in the N-amended plots. This decline in soil respiration has not been explained by other factors, that is, root respiration or microbial respiration in mineral soil (Burton *et al.*, 2004; Zak *et al.*, 2006). Furthermore the N-amended plots have shown increased tree growth (K. S. Pregitzer *et al.*, unpublished), which suggests that more C is invested in above-ground biomass.

Another hypothesis for reduced AM fungal biomass with N addition could be that the mycorrhizas are directly affected by the higher amounts of N in the soil. Wallander (1995) suggested that reduced fungal growth was not caused by reduced C flow to ectomycorrhizal fungi (EMF), but that the increased amount of N supply caused the mycorrhizal fungi to use more C in the costly process of N assimilation instead of using the C for growth. This hypothesis is consistent with the increased N content of the foliage and leaf litter of the N-amended plots at our study sites (K. S. Pregitzer, unpublished). Fungal growth response can also differ among species depending on their capacity for N assimilation and the pathway of N assimilation (Wallander, 1995), and this might also explain some of the differences in treatment response among sites. However, N uptake costs may be lower for AMF than EMF, because of the difference in their N-assimilation pathways. In AMF symbioses studied so far, N is transferred to the host plant as ammonium and not, as in EM symbiosis, as an amino acid (Govindarajulu *et al.*, 2005). Therefore AMF retain most of the C from the amino acids, while EM fungi lose the C in the transfer of N as amino acids to the host plant (Govindarajulu *et al.*, 2005). However, the N-uptake by AMF still has energetic and C costs that could affect AMF growth.

Sites varied in the strength of the reduction in AMF colonization with N addition. Site D showed only a marginal decline in AMF abundance with N addition in July and a trend toward an increase in October. Although the ambient N deposition of site D is about the same as that of site C, the lack of strong reduction of AM fungal root abundance with N addition could possibly be caused by site-level differences in C allocation to, or N-assimilation by, AMF caused by variation in mean annual temperature, precipitation, tree growth, N-mineralization rates, C : N ratio in litter or phosphorus availability. Alternatively, site-level differences in AMF abundance could be driven by changes in AM fungal community structure. Functional diversity (e.g. variation in C demand vs nutrient supply) exists among AMF, and compositional and functional community responses have been found in previous studies of AM fungal response to N ( Johnson, 1993; Corkidi *et al.*, 2002). For example, Johnson (1993) found a change in AM fungal community with N (and other nutrients)

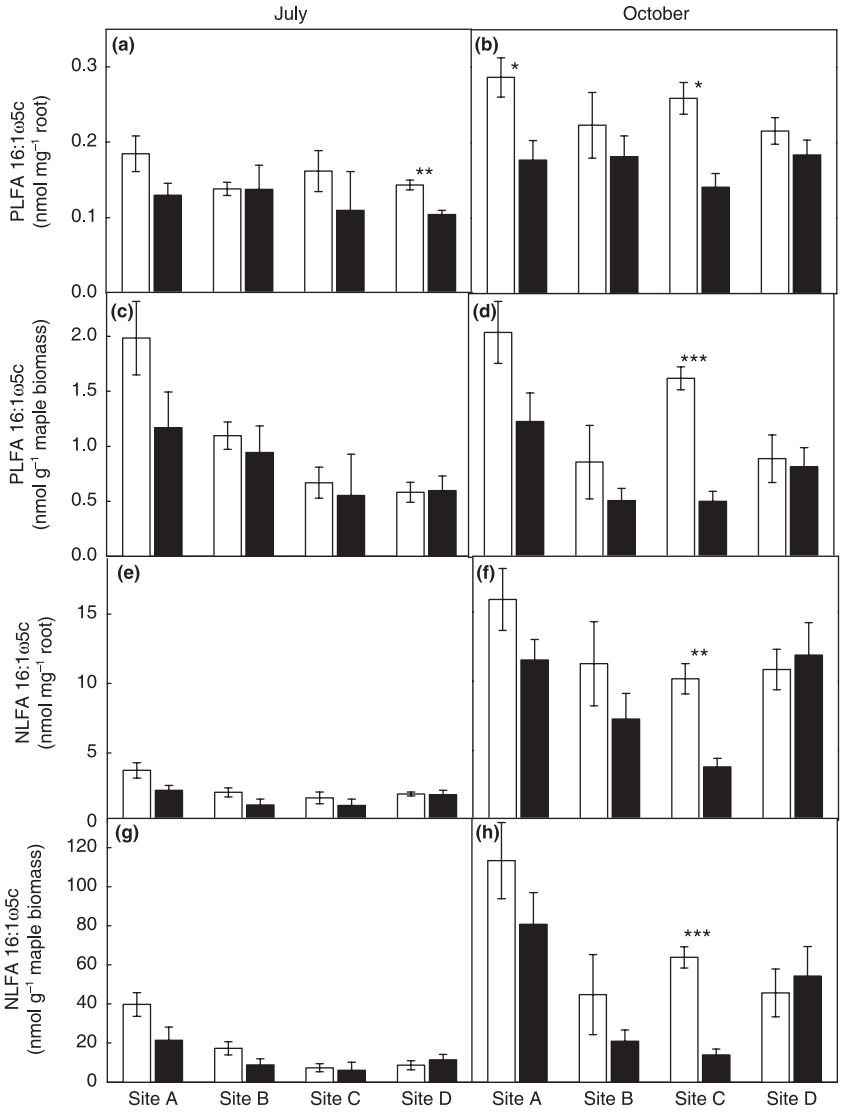


**Fig. 3** Mean phospholipid fatty acid (PLFA) (a, b) and neutral lipid fatty acid (NLFA) 16:1ω5c (e, f) concentrations in maple (*Acer* spp.) fine roots, and maple proportional allocation to PLFA (c, d) and NLFA 16:1ω5c (g, h), for the four study sites by treatment (open bars, control; closed bars, N-amended) for July and October 2005. Error bars indicate 1 SE of the mean. Overall N treatment effect was significant in all cases (see Table 1 for details). Means of N treatments differ significantly at site: *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$.

fertilization and suggested that the AM fungal species dominant at the fertilized sites were more parasitic than those dominant at low-N sites. We will address these alternative hypotheses in a future paper.

## Staining vs fatty acid methods

The positive linear relationship of the fatty acid 16:1ω5c with percentage AMF colonization in stained roots found in this study is consistent with findings from other studies that have performed both staining and fatty acid analysis (Olsson *et al.*, 1997; Van Aarle & Olsson, 2003; R. M. Miller, unpublished). In a controlled glasshouse study with cucumber plants inoculated with a single AMF species, very strong relationships were found between colonized root length and both PLFA and NLFA 16:1ω5c ($R^2 = 0.92$ and 0.95, respectively) (Olsson *et al.*, 1997). In another glasshouse study, Van Aarle & Olsson (2003) found weaker significant relationships between both PLFA and NLFA 16:1ω5c and percentage AMF colonization ($R^2 = 0.44$ and 0.57, respectively). The higher $R^2$ values within the Olsson *et al.* (1997) and the Van Aarle & Olsson (2003) study compared with our study could be caused by the much more controlled environment vs a field study; a single AMF species vs greater AMF diversity combined with differences between AMF species in fatty acid composition and amounts (Bentivenga & Morton, 1996; Olsson & Johansen, 2000); and/or a bigger range and better distribution of the values of root colonization.

The relationship of NLFA 16:1ω5c with the amount of storage structures (Fig. 4c) was stronger than that of PLFA 16:1ω5c with the percentage total AMF colonization (Fig. 4a). It is unclear exactly why this is, but possibilities include the inability to distinguish live and dead hyphae using staining methods; poor staining of some AMF species

 

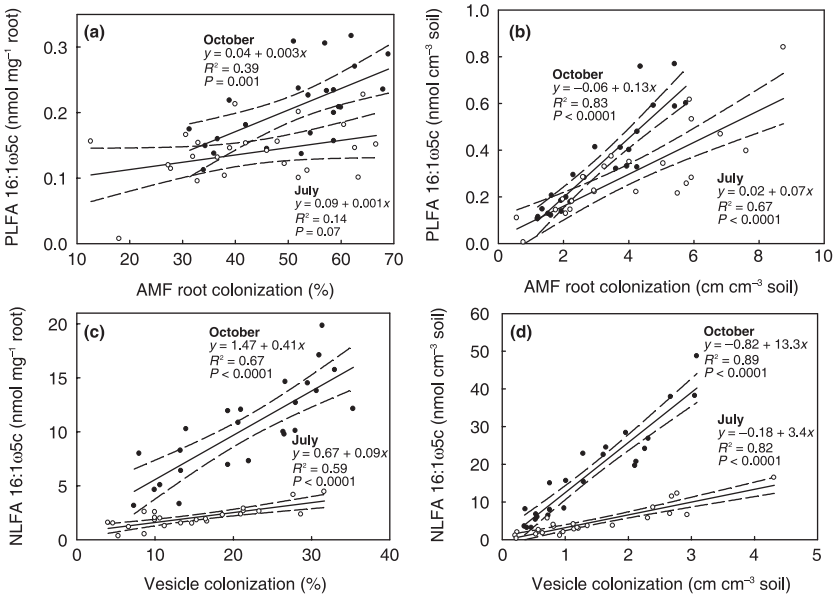


**Fig. 4** Relationship between phospholipid fatty acid (PLFA) 16:1ω5c and total arbuscular mycorrhizal fungal (AMF) colonization (a, b), and between neutral lipid fatty acid (NLFA) 16:1ω5c and vesicle colonization (vesicles and intraradical spores) (c, d) in maple (*Acer* spp.) fine roots for the four study sites as colonization intensity (a, c) and on maple stand-level basis (b, d). Open circles, July; closed circles, October. Regression line (solid) and 95% confidence interval (dashed) are plotted.

(Morton & Redecker, 2001); vesicles' larger size and distinctive shape compared with hyphae, which minimizes error in counting; and the larger potential for variability in hyphal density compared with vesicle density at an intersect.

The steeper slope of the relationship between NLFA 16:1ω5c and vesicle colonization in October compared with July is indicative of vesicle filling, that is, the accumulation of storage lipids through the growing season. This suggests that most of the AMF storage structures (vesicles) are already present earlier on in the colonization process of the roots, and more lipids are added to these vesicles during the growing season for storage and use for the next year. A similar observation was made by Van Aarle & Olsson (2003) in their glasshouse study. NLFA 16:1ω5c is therefore perhaps a better indicator of the amount of stored energy than the numbers of vesicles present in the roots.

We saw a similar, but weaker, effect of season on the relationship of PLFA 16:1ω5c and percentage total AMF colonization. The distinction between the two regression lines in this relationship (Fig. 4a) is less obvious than for NLFA 16:1ω5c vs vesicle colonization (Fig. 4c). The threefold steeper slope in October compared with July is a much smaller relative increase compared with the fivefold steeper slope for the neutral lipids vs percentage vesicles. PLFA 16:1ω5c also appeared to be a more sensitive biomass indicator than our frequency-based ocular measurements of AMF colonization, probably because the ocular method does not take colonization intensity into account. As a result, when only ocular measurements are performed, changes in biomass could be overlooked or underestimated.

The improvement of the relationship of lipid and ocular estimates after rescaling to a volumetric ($cm^3$ soil) basis was striking, indicating that the strength of the relationship of the two metrics depends on the form of their expression. Since mycorrhizas and roots exploit space rather than mass, the stand-level values (Fig. 4b,d), which show the actual mycorrhizal biomass in a volume of soil, are perhaps more relevant to use than concentration values (Fig. 4a,c). Both root biomass and specific root length, which were used to calculate AM fungal biomass on a stand-level basis, were not affected by treatment. However, the percentage colonization decreased with an increase in specific root length ($R^2 = 0.47$, $P < 0.0001$) and root biomass decreased at all sites from July to October ($P = 0.001$). By expressing the AMF abundance on a volumetric basis, these length and biomass differences were taken into account, and improved the relationships between ocular measurements and fatty acid 16:1ω5c.

In conclusion, after 12 yr of simulated N-addition, the abundance of AMF within the active fine root system of maples and proportional investment in AMF decreased significantly, as estimated by both lipid analysis and staining. Positive linear relationships were found between the fatty acid 16:1ω5c and the percentage total AMF colonization and number of storage structures. The phospholipid fraction seems to be a good indicator of active AMF biomass, and NLFA 16:1ω5c was found to be a better indicator of AMF stored energy than the number of vesicles present. The fatty acid analyses gave a better insight into changes in AMF total biomass and stored energy over time compared with the staining method, and avoided possible under- or overestimation of the total AM fungal abundance. However, the staining method can elucidate changes in specific fungal structures (arbuscules, coils, etc.), which is not possible with fatty acid analyses. The observed decrease in AMF abundance and investment could suggest either reduced C allocation to these fungi or a direct soil N-mediated decline. The observed

reduction in the abundance of, and investment in, AMF below ground is consistent with the reduction in soil respiration reported earlier for this study (Burton *et al.*, 2004). Future research will focus on the effects of increased N inputs on AMF extraradical hyphae and community analyses designed to understand if N deposition is altering AMF community composition, structure and function.

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